

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/20212

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/567; A01N 37/18; A61K 38/00

US CL :435/7.21, 514/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.21, 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,385,915 (BUXBAUM ET AL) 31 January 1995, see entire document.	3
X	US 5, 538,983 (BUXBAUM ET AL) 23 July 1996, see entire document.	3
Y	US 5,262,332 (SELKOE) 16 November 1993, see entire document.	1, 2
Y	US 5,547,841 (MAROTTA ET AL) 20 August 1996, see column 3, lines 7-14 and claims 1-3.	1, 2

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

29 JANUARY 1998

Date of mailing of the international search report

04 MAR 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Washington, D.C. 20231

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PATRICIA A. DUFFY

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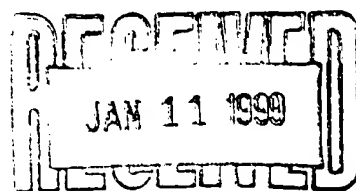
INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/20212

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HAASS et al. The Swedish mutation causes early-onset Alzheimer's disease by beta-secretase cleavage within the secretory pathway. Nature Medicine, December 1995, Vol. 1, No. 12, pages 1291-1296, see entire document.	1, 2
Y	WERTKIN et al. Human neurons derived from a teratocarcinoma cell line express solely the 695-amino acid amyloid precursor protein and produce intracellular beta-amyloid or A4 peptides. Proc. Natl. Acad. Sci. USA, October 1993, Vol. 90, pages 9513-9517, see entire document.	1, 2
Y	TURNER et al. Amyloids beta40 and beta42 are generated intracellularly in cultured human neurons and their secretion increases with maturation. The Journal of Biological Chemistry, 12 April 1996, Vol. 271, No. 15, pages 8966-8970, see entire document.	1, 2
Y	MARTIN et al. Intracellular accumulation of beta-amyloid in cells expressing swedish mutant amyloid precursor protein. The Journal of Biological Chemistry, 10 November 1995, Vol. 270, No. 45, pages 26727-26730, see entire document.	1, 2

PATENT COOPERATION TREATY



From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To: JANE MASSEY LICATA
LAW OFFICES OF JANE MASSEY LICATA
66 E. MAIN STREET
MARLTON, NEW JERSEY 08053

Docket System ✓
Status Report ✓
Docket Book ✓

5/15/99

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing
(day/month/year)

05 JAN 1999

Applicant's or agent's file reference

PENN-0561

IMPORTANT NOTIFICATION

International application No.

PCT/US97/20212

International filing date (day/month/year)

13 NOVEMBER 1997

Priority Date (day/month/year)

15 NOVEMBER 1996

Applicant

THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.
4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US
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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference PENN-0561	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US97/20212	International filing date (day/month/year) 13 NOVEMBER 1997	Priority date (day/month/year) 15 NOVEMBER 1996
International Patent Classification (IPC) or national classification and IPC IPC(6): G01N 33/567; A01N 37/18; A61K 38/00 and US Cl.: 435/7.21, 514/2		
Applicant THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand 09 JUNE 1998	Date of completion of this report 16 DECEMBER 1998
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PATRICIA A. DUFFY Telephone No. (703) 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US97/20212

I. Basis of the report

1. This report has been drawn on the basis of *(Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments);*

- ☒ the international application as originally filed.
- ☒ the description, pages 1-20 , as originally filed.
pages NONE , filed with the demand.
pages NONE , filed with the letter of _____.
pages _____ , filed with the letter of _____.
- ☒ the claims, Nos. 1-3 , as originally filed.
Nos. NONE , as amended under Article 19.
Nos. NONE , filed with the demand.
Nos. NONE , filed with the letter of _____.
Nos. _____ , filed with the letter of _____.
- ☒ the drawings, sheets/~~fig~~ NONE , as originally filed.
sheets/~~fig~~ NONE , filed with the demand.
sheets/~~fig~~ NONE , filed with the letter of _____.
sheets/~~fig~~ _____ , filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

- ☒ the description, pages NONE .
- ☒ the claims, Nos. NONE .
- ☒ the drawings, sheets/~~fig~~ NONE .

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**1. STATEMENT**

Novelty (N)	Claims <u>1, 2</u>	YES
	Claims <u>3</u>	NO
Inventive Step (IS)	Claims <u>2</u>	YES
	Claims <u>1, 3</u>	NO
Industrial Applicability (IA)	Claims <u>1-3</u>	YES
	Claims <u>NONE</u>	NO

2. CITATIONS AND EXPLANATIONS

Claim 3 lacks novelty under PCT Article 33(2) as being anticipated by Bauxbaum et al (U.S. Patent 5,385,915).

Bauxbaum et al teach the treatment of Alzheimer's disease using modulators of protein kinase activity (see column 28, claims 21-26). In particular Bauxbaum et al teach that compounds (i.e. okadaic acid or PDBu) which stimulate APPs production, also inhibit beta amyloid peptide production (see column 24, lines 41-57). Thus, Bauxbaum et al teach that these compounds can be used to slow the development of Alzheimer's Disease. The compounds of Bauxbaum et al tested in neural cells are identifiable by the method of claim 1. As a result, Bauxbaum et al anticipates the instantly claimed invention.

Applicants' argue that the compounds identified by Bauxbaum et al were not identified by the method of claim 1 which is directed to measurement of peptides in the endoplasmic reticulum and thus do not anticipate the instant method. This is not persuasive, different reagents which act the same can be identified by different methods. Applicants' have not shown that the reagents of Bauxbaum et al do not function in the assay of claim 1 or do not inhibit beta amyloid peptide production. Thus, the agents of Bauxbaum et al could have been identified by the method of claim 1. Applicants' have not provided any evidence that the reagents of the prior art do not function to block peptide production. Applicants' arguments are not convincing.

Claim 3 lacks novelty under PCT Article 33(2) as being anticipated by Gandy et al (U.S. Patent 5,242,932).

Gandy et al teach the modulating or affecting the intracellular trafficking and processing of amyloid precursor protein in the cell of a patient in need of therapy for amyloidosis associated with Alzheimer's disease using modulators of APP processing (see columns 13-14, claims 1-8). In particular Bauxbaum et al teach that compounds (i.e. chloroquine, monesin, brefeldin A) inhibit secretion of amyloid precursor protein fragments such as beta amyloid (see paragraph bridging columns 12-13). Thus, Gandy et al teach that these compounds can be used to slow the development of Alzheimer's Disease. The compounds of Gandy et al (i.e. brefeldin A) would have been products (Continued on Supplemental Sheet.)

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

The description is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 5 because it fails to adequately enable practice of the claimed invention because: the disclosure fails to provide a written description of even one agent which is diagnostic for Alzheimer's disease and increases the production of amyloid in the endoplasmic reticulum of NT2N cells. No biological agents are set forth at all. The disclosure provides no guidance as which biological agents to test.

Applicants' argue that no claims are drawn to an agent. The examiner respectfully disagrees, as clearly claims 2 uses a diagnostic agent to diagnose disease. There is no written description of a single agent identified by claim 1 which functions appropriately in claim 1 and functions as a diagnostic agent for disease in claim 2. Applicants' arguments are not persuasive the disclosure is not enabled for this claim.

Claim 2 is objected to as lacking clarity under PCT Rule 66.2(a)(v) because practice of the claimed invention is not enabled as required under PCT Rule 5.1(a) for the reasons set forth in the immediately preceding paragraph.

Claims 2 and 3 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because the claims are drawn to completely different methods and are indefinite for the following reason(s): Claim 1 is drawn to a method for screening for agents which increase or decrease the production of amyloid in an in vitro cell system. Claim 2 is drawn to a method of diagnosis using an agent identified by the process of claim 1 which increases amyloid peptide production by the method of claim 1. However, the method of claim 1 is a screening method, no agents are identified, therefore one could not diagnose disease because one skilled in the art has no idea of what agent to detect for diagnostic purposes. Claim 3, is drawn to a method of treatment of Alzheimer's disease using an agent which inhibits the production of amyloid peptides identified by the process of claim 1. However, the method of claim 1 is a screening method, no agents are identified, therefore one could not treat disease because one skilled in the art has no idea of what agent to administer for therapeutic purposes.

Applicants' essentially argue that description of diagnostic or therapeutic agents is not required because all that would need to be done is to perform the method of claim 1. This is not persuasive, claim 1 provides no structure for any diagnostic or therapeutic agent. Moreover, no therapeutic or diagnostic agent is disclosed which has diagnostic or therapeutic use which has been identified by the method of claim 1. Merely because one might be able to perform claim 1, this does not indicate that the agents which modulate processing are either therapeutic or (Continued on Supplemental Sheet.)

Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):

identified by the method of claim 1. As a result, Gandy et al anticipates the instantly claimed invention.

Applicants' argue that the compounds identified by Bauxbaum et al were not identified by the method of claim 1 which is directed to measurement of peptides in the endoplasmic reticulum and thus do not anticipate the instant method. This is not persuasive, different reagents which act the same can be identified by different methods. Applicants' have not shown that the reagents of Bauxbaum et al do not function in the assay of claim 1 or do not inhibit beta amyloid peptide production. Thus, the agents of Bauxbaum et al could have been identified by the method of claim 1. Applicants' have not provided any evidence that the reagents of the prior art do not function to block peptide production. Moreover, brefeldin A is one agent which applicants' have disclosed which blocks endoplasmic processing. Thus, applicants' arguments are not convincing.

Claim 1 lacks an inventive step under PCT Article 33(3) as being obvious over Marotta et al (U.S. Patent 5,547,841) in view of Turner et al (The Journal of Biological Chemistry 12 April 1996).

Marotta et al teach the cells lines expressing the amyloid gene are that they are useful to screen for drugs that are potentially useful for treating Alzheimer's Disease by stopping the production of amyloid.

Turner et al teach that human NT2N cells produce intracellular amyloid peptide and that the peptide can be detected using monoclonal antibodies (see entire document, abstract in particular).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to use the intracellular amyloid peptide assay system of Turner et al to screen for agents that modulate amyloid peptide production because Marotta et al teach that cells lines expressing the amyloid gene are that they are useful to screen for drugs that are potentially useful for treating Alzheimer's Disease by stopping the production of amyloid.

Applicants' argue that the art does not teach the detection of intracellular amyloid peptide in the endoplasmic reticulum. The disclosure like the art uses immunoprecipitation of whole cell lysates and cell culture supernatants. Thus, the method of detection when read in light of the disclosure does not distinguish from the method of detection in the art. The methods used to detect and quantitate the amyloid peptide in the art properly detects the amyloid peptide in the endoplasmic reticulum.

Claim 2 meets the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest the claimed invention because the examiner can not ascertain the "agent" which is diagnostic, no agents have been identified in the disclosure which are diagnostic.

NEW CITATIONS

US 5,242,932 A (GANDY et al.) 07 September 1993.

VIII. CERTAIN OBSERVATIONS ON THE APPLICATION (Continued):

diagnostic. Applicants' have not disclosed other accepted therapeutic or diagnostic agents in the art have the ability to be identified by the method of claim 1. Thus, the claims remain indefinite.

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

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in its capacity as elected Office

Date of mailing (day/month/year)

07 July 1998 (07.07.98)

International application No.

PCT/US97/20212

Applicant's or agent's file reference

PENN-0561

International filing date (day/month/year)

13 November 1997 (13.11.97)

Priority date (day/month/year)

15 November 1996 (15.11.96)

Applicant

LEE, Virginia, M.-Y. et al

1. The designated Office is hereby notified of its election made:



in the demand filed with the International Preliminary Examining Authority on:

09 June 1998 (09.06.98)



in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was

was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
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1211 Geneva 20, Switzerland

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Eugénia Santos

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : G01N 33/567, A01N 37/18, A61K 38/00	A1	(11) International Publication Number: WO 98/21589 (43) International Publication Date: 22 May 1998 (22.05.98)
(21) International Application Number: PCT/US97/20212 (22) International Filing Date: 13 November 1997 (13.11.97) (30) Priority Data: 60/030,967 15 November 1996 (15.11.96) US 60/055,299 8 August 1997 (08.08.97) US (71) Applicant (for all designated States except US): THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Center for Technology Transfer, Suite 300, 3700 Market Street, Philadelphia, PA 19104-3147 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): LEE, Virginia, M.-Y. [US/US]; 2005 Pine Street, Philadelphia, PA 19103 (US). DOMS, Robert, W. [US/US]; 1230 Leopard Road, Berwyn, PA 19312 (US). (74) Agents: LICATA, Jane, Massey et al.; Law Offices of Jane Massey Licata, 66 E. Main Street, Marlton, NJ 08053 (US).		(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: SCREENING FOR MODULATORS OF AMYLOID PROCESSING (57) Abstract <p>β secretase pathways which process amyloid precursor protein into the amyloid peptides of neuritic plaques and vascular deposits that accumulate in the brains of patients with Alzheimer's disease have been identified in the endoplasmic reticulum of neuronal cells. Methods of identifying compounds which increase or decrease processing of amyloid precursor protein via these pathways which may be useful in the treatment of Alzheimer's disease and in identifying causative agents of this disease are provided.</p>		

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SCREENING FOR MODULATORS OF AMYLOID PROCESSING

Introduction

5 This invention was made in the course of research sponsored by the National Institutes of Health. The U.S. Government may have certain rights in this invention.

Field of the Invention

10 Patients suffering from Alzheimer's disease are afflicted with neuritic plaques and vascular deposits consisting of amyloid fibrils made up of amyloid β peptides. Novel β -secretase enzymatic pathways which process amyloid β precursor peptide to amyloid β peptides have now been identified and are located in the endoplasmic reticulum. The
15 present invention relates to methods of identifying agents which increase or decrease processing of amyloid precursor protein into amyloid β peptides by contacting NT2N cells with the agent and measuring levels of amyloid β peptides formed in the endoplasmic reticulum of the cells. Agents identified to
20 increase processing of amyloid precursor protein into amyloid β peptides can be used in the early diagnosis of Alzheimer's disease while agents identified to decrease this processing are
25 expected to be useful in the treatment of Alzheimer's disease.

Background of the Invention

25 Amyloid β ($A\beta$) peptides are the building blocks of the amyloid fibrils found in neuritic plaques and vascular deposits

that accumulate in the brains of patients with Alzheimer's disease (AD; Selkoe, D. J. 1994 *Ann. Rev. Cell. Biol.* 10:373-403). $A\beta$ is derived from proteolytic processing of one or more isoforms of the amyloid precursor protein (APP; Kang et al. 1987 *Nature* 325:733-736). APP isoforms are alternatively spliced type I transmembrane glycoproteins that are encoded by a single gene on human chromosome 21 (Kang et al. 1987 *Nature* 325:733-736; St. George-Hyslop et al. 1987 *Science* 235:885-890). The 39-43 amino acid long $A\beta$ sequence begins in the ectodomain of APP and extends into the transmembrane region. Of the 3 major $A\beta$ -containing isoforms encoded by the APP gene (i.e., APP695, APP751, and APP770; Kang et al. 1987 *Nature* 325:733-736; Ponte et al. 1988 *Nature* 331:525-527; Tanzi et al. 1988 *Nature* 331:528-530; Kitaguchi et al. 1988 *Nature* 331:530-532), APP695 is expressed almost exclusively by neurons of the central and peripheral nervous systems (Golde et al. 1990 *Neuron* 4:253-267; Kang, J. and Müller-Hill, B. 1990 *Biochem. Biophys. Res. Commun.* 166:1192-1200; Arai et al. 1991 *Ann. Neurol.* 30:686-693).

Newly synthesized APP matures in the endoplasmic reticulum and the Golgi apparatus acquiring N- and O-linked carbohydrates, tyrosine sulfates (Weidemann et al. 1997 *Nature Med.* 3:328-332; Oltersdorf et al. 1990 *J. Biol. Chem.* 265:4492-4497) and phosphates (Oltersdorf et al. 1990 *J. Biol. Chem.* 265:4492-4497; Suzuki et al. 1992 *Neurosci.* 48:755-761; and Knops et al. 1993 *Biochem Biophys. Res. Comm.* 197:380-385). Several pathways of APP metabolism have been described in cultured cells, and evidence suggests that the relative importance of each pathway depends on the cell type. For example, non-neuronal cells preferentially process APP by the α -secretase pathway which cleaves APP within the $A\beta$ sequence, thereby precluding the formation of $A\beta$ (Esch et al. 1990 *Science* 248:1122-1124; Sisodia et al. 1990 *Science* 248:492-495). The putative α -secretase enzyme(s) is active at or near the cell surface, causing the N-terminal fragment ($APP\alpha$) to be quickly secreted. In contrast, neuronal cells process a much larger portion of APP by the β -secretase pathway(s), which

generates intact $A\beta$ by the combined activity of two enzyme classes. The β -secretase(s) cleaves APP at the amino terminus of the $A\beta$ domain releasing a distinct N-terminal fragment ($APP\beta$). In addition, the γ -secretase(s) cleaves APP at
5 alternative sites of the carboxy terminus generating species of $A\beta$ that are either 40 ($A\beta_{40}$) or 42 amino acids long ($A\beta_{42}$; Seubert et al. 1993 *Nature* 361:260-263; Suzuki et al. 1994 *Science* 264:1336-1340; Turner et al. 1996 *J. Biol. Chem.* 271:8966-8970).

10 $A\beta_{42}$ is believed to have a significant role in AD pathology. Studies have shown that $A\beta_{42}$ is more prone to formation of insoluble aggregates. Jarrett et al. 1993 *Cell* 73:1055-1058. Further, mutations in APP which increase the relative proportion of $A\beta_{42}$ have been linked to familial AD.
15 Suzuki et al. 1994 *Science* 264:1336-1340. In addition, $A\beta_{42}$ has been shown to be preferentially deposited in amyloid plaques. Iwatsubo et al. 1994 *Neuron* 13:45-53.

In vitro studies indicate the existence of at least two β -secretase pathways. In the endosomal/lysosomal pathway,
20 APP targeted to the cell surface is endocytosed and delivered to endosomes and lysosomes where β - and γ -cleavages can occur (Golde et al. 1992 *Science* 255:728-730; Nordstedt et al. 1993 *J. Biol. Chem.* 268:608-612; Haass et al. 1992a *Nature* 357:500-503; Koo, E. H. and Squazzo, S. 1994 *J. Biol. Chem.* 269:17386-
25 17389; Lai et al. 1995 *J. Biol. Chem.* 270:3565-3573; Perez et al. 1996 *J. Biol. Chem.* 271:9100-9107). The alternative β -secretory pathway is believed to generate $A\beta$ in Golgi-derived vesicles, most likely secretory vesicles, prior to secretion (Haass et al. 1995a *Nature Med.* 1:1291-1296; Higaki et al. 1995
30 *Neuron* 14:651-659; Perez et al. 1996 *J. Biol. Chem.* 271:9100-9107; Thinakaran et al. 1996b *J. Biol. Chem.* 271:9390-9397).

Both $A\beta_{40}$ and $A\beta_{42}$ have been shown to be produced intracellularly from endogenous wild-type APP695 by cultured
postmitotic CNS neuronal cells (NT2N) that are induced to
35 differentiate from a human teratocarcinoma cell line (NT2) by treatment with retinoic acid (Pleasure et al. 1992 *J. Neurosci.* 12:1802-1815; Pleasure, S. J. and Lee, V. M.-Y. 1993 *J.*

Neurosci. Res. 35:585-602; Wertkin et al. 1993 *Proc. Natl. Acad. Sci. USA* 90:9513-9517; Turner et al. 1996 *J. Biol. Chem.* 271:8966-8970). To date, the human derived NT2N neuron is the only cell line documented to generate intracellular $A\beta_{40}$ and $A\beta_{42}$ before their eventual release into the medium (Turner et al. 1996 *J. Biol. Chem.* 271:8966-8970). Because neurons are the cell type most adversely affected by AD, the NT2N neurons represent a unique system for the study of APP processing and $A\beta$ biogenesis. An essential first step in the analysis of such pathways is the identification of the proteolytic fragments that are the products of these cleavages.

It has now been found that γ -secretase acts in the Endoplasmic Reticulum (ER) to yield $A\beta_{42}$. The N-terminal fragment generated by β -cleavage (i.e., $APP\beta$) has also been found to be produced by β -secretase intracellularly in NT2N neurons prior to secretion. These protease activities were identified to occur in the Endoplasmic Reticulum (ER)/Intermediate Compartment (IC) of neuronal cells utilizing inhibition with Brefeldin A (BFA), incubation at 15°C, and expression of exogenous APP bearing the di-lysine ER-retrieval motif. Accordingly, the present invention relates to methods of identifying compounds targeted to the endoplasmic reticulum which increase or decrease processing of amyloid precursor protein into amyloid β peptides found in neuritic plaques and vascular deposits that accumulate in brains of patients with Alzheimer's disease.

Summary of the Invention

Novel β -secretase pathways have now been identified that produce amyloid β peptides, $A\beta_{42}$ and $APP\beta$, in the endoplasmic reticulum/intermediate compartment. Discovery of these enzymatic pathways and their location within the endoplasmic reticulum can be used to design new therapeutic approaches or agents which reduce production of amyloid β peptides. An object of the present invention is to provide a method of identifying agents which modulate processing of amyloid precursor protein into amyloid β peptides found in

neuritic plaques and vascular deposits that accumulate in brains of patients with Alzheimer's disease. In one embodiment, the method of the present invention may be used to identify inhibitors of this process which may be useful in the treatment of Alzheimer's disease. Alternatively, the method of the present invention may be useful in identifying agents or genetic mutations which increase this process, thereby increasing the formation of amyloid β peptides and the possibility of developing Alzheimer's disease.

10 Detailed Description of the Invention

APP serves as a substrate for a variety of proteolytic processing pathways, only some of which result in the production of $A\beta$ (Selkoe, D. J. 1994 *Ann. Rev. Cell. Biol.* 10:373-403). However, $A\beta$ is the major component of senile plaques in the AD brain. Moreover, mutations in the APP gene associated with Familial Alzheimer's Disease (FAD) alter APP processing and $A\beta$ production *in vitro* (Citron et al. 1992 *Nature* 360:672-674; Cai et al. 1993 *Science* 259:514-516; Suzuki et al. 1994 *Science* 264:1336-1340). Thus, determination of the proteolytic events that lead to $A\beta$ production and identification of the proteases responsible for each step as well as the sites of their action are important in the development of treatments for Alzheimer's disease and in the identification of causes for this disease.

25 In the present invention, the NT2N system was used to study APP processing in neurons. NT2N cells have been reported produce intracellular $A\beta$ (Wertkin et al. 1993 *Proc. Natl. Acad. Sci. USA* 90:9513-9517; Turner et al. 1996 *J. Biol. Chem.* 271:8966-8970). NT2N neurons express the isoform of APP expressed almost exclusively in the CNS (i.e., APP695) and generate detectable intracellular levels of both $A\beta_{40}$ and $A\beta_{42}$. Further, they constitutively produce and secrete $A\beta$. Using antibodies specific to APP β and other proteolytic fragments, a number of intracellular β -secretase activities which cleave on the amino terminus side of $A\beta$ have now been identified and characterized. Moreover, novel β -secretase activities that

occur in a pre-Golgi compartment have now been identified.

For example, β -secretase(s) cleaves APP at the amino terminus of the A β domain releasing a distinct N-terminal fragment (APP β). To determine if intracellular APP β could be recovered from NT2N cells, samples of cell lysate were immunoprecipitated with Karen (an antiserum raised to the N-terminal region of APP). The presence of APP β in the immunoprecipitate was determined by immunoblot analysis using 53 (a polyclonal antibody specific for the free carboxy-terminus of APP β). This polyclonal antibody detected a single band of approximately 95 kDa. The identity of this 95 kDa APP fragment to be APP β cleaved at the β -secretase site was confirmed by: 1) the inability of 369W, an antibody specific for the C-terminus of APP, to recognize this fragment; 2) the inability of 6E10, an antibody specific for the first 10 amino acid residues of A β , to detect this fragment; 3) the binding of Karen, an antibody that recognizes all APP species, to this fragment; 4) the fact that this intracellular APP fragment is about 11-12 kDa smaller than APP_{FL}; and 5) the detection of the same 95 kDa APP fragment using a different antibody specific for APP β (i.e., 192; Seubert et al. 1992 *Nature* 359:325-327). APP β was readily detected in the media of NT2N neurons and co-migrated with APP β recovered from the cell lysates thus indicating that APP β was secreted.

The detection of intracellular APP β and A β in NT2N neurons indicates that β -secretase pathway including β - and γ -secretase occur in an intracellular compartment. The absence of intracellular APP α , however, suggests that the majority or all of the α -secretase activity occurs at a different site. To further confirm that the β -secretase pathways, but not the α -secretase pathway, occur inside these cells, the cell lysate of NT2N neurons was examined for the products of these respective pathways: A β , which is generated by β - and γ -secretase cleavages; and p3, a product of α - and γ -secretase cleavages. In these experiments, cell lysates of metabolically labeled NT2N neurons were immunoprecipitated with monoclonal antibodies (mAb) that can distinguish between these peptides: 4G8

recognizes both A β and p3; Ban50, however, binds only to A β and not p3. Data from these experiments clearly demonstrates that A β , but not p3, is produced intracellularly. The p3 fragment was not detected in cell lysates even after prolonged exposure
5 of the film. By contrast, both A β and p3 were readily recovered from the media.

A series of experiments were performed which confirm that APP β is derived from APP_{FL} within the cell prior to secretion. In a first set of experiments, APP β was recovered
10 from NT2N cell lysates even after intact NT2N neurons were treated with trypsin. Cultures of NT2N neurons were treated with trypsin at 4°C. Under such conditions, cell surface-associated but not intracellular APP β should be proteolyzed. However, a similar amount of APP β was recovered from NT2N
15 neurons regardless of trypsin treatment. By contrast, when the NT2N neurons were treated with trypsin and 0.1% Triton X-100, intracellular APP β was completely eliminated. Thus APP β recovered from the NT2N cell lysate is indeed produced in an intracellular compartment.

20 A second set of experiments confirmed the continuous presence of steady state levels of APP β in NT2N neurons, together with a delay in the detection of APP β in freshly replenished medium. In these experiments, NT2N neurons were washed with fresh medium and the amount of intracellular as
25 well as secreted APP β and APP α were measured over an 8 hour period by immunoprecipitation of cell lysates and media with Karen followed by immunoblotting with either antibody 53 (for APP β) or 6E10 (for APP α). Secreted APP β was first detected in 3 to 5 hours, and its accumulation in the medium continued over
30 the 8 hour incubation period. In contrast, APP α was detected in one hour, suggesting that APP α is produced at a faster rate than APP β . As seen with APP β , APP α accumulated in the conditioned media over time. A steady state level of APP β was recovered from NT2N cell lysates prepared from parallel
35 cultures over a period of 8 hours this indicating that intracellular APP β is produced constitutively.

Pulse-chase experiments were also performed which

demonstrated that the turnover of intracellular APP β lags behind the turnover of newly synthesized APP_{FL}, thereby confirming that APP β is generated from APP_{FL} inside NT2N neurons before secretion. This pulse-chase paradigm permits a more rigorous study of the temporal relationship between intracellular and secreted APP β . In these experiments, NT2N cultures were pulsed with [³⁵S]methionine for one hour and then chased for different lengths of time. After one hour of chase time, full length APP (APP_{FL}) immunoprecipitated from the cell lysate began to decline while the intracellular level of APP β continued to increase until 4 hours, after which it also declined. This lag in maximum production of intracellular radiolabeled APP β indicates that APP β is produced intracellularly from APP_{FL} by β -secretase cleavage. Further, the one hour delay in the secretion of APP β into the medium as well as the accumulation of this fragment with increasing chase time supports a temporal relationship between APP β that is produced intracellularly and APP β that is secreted into the medium.

The detection of APP β in the cell lysate of NT2N neurons, together with the presence of A β ₄₀ and A β ₄₂ (Turner et al. 1996 *J. Biol. Chem.* 271:8966-8970), establish that an intracellular β -secretase pathway(s) exists in these cells. At present, no other cell line has been reported to produce detectable levels of intracellular APP β from endogenous or over-expressed wild-type APP (Seubert et al. 1993 *Nature* 361:260-263; Haass et al. 1995a *Nature Med.* 1:1291-1296; Thinakaran et al. 1996b *J. Biol. Chem.* 271:9390-9397). Only human kidney 293 cells stably transfected with APP_{sw} cDNA yield the related APP β _{sw} fragment from the cell lysates (Haass et al. 1995a *Nature Med.* 1:1291-1296; Martin et al. 1995 *J. Biol. Chem.* 270:26727-26730). In these non-neuronal cells, however, treatment with BFA completely eliminates APP β _{sw} and A β production (Haass et al. 1995a *Nature Med.* 1:1291-1296; Martin et al. 1995 *J. Biol. Chem.* 270:26727-26730; Essalmani et al. 1996 *Biochem. Biophys. Res. Commun.* 218:89-96). In contrast, NT2N neurons continue to produce APP β and A β during treatment

with BFA, implying that the subcellular site(s) of the β -secretase pathway is cell-type specific. In these experiments, NT2N neurons were metabolically labeled with [35 S]methionine in the presence or absence of 20 μ g/ml Brefeldin A (BFA). BFA is a pharmacological agent that causes a redistribution of the Golgi into the ER. In the absence of BFA, APP_{FL}, APP β , and A β were recovered from the cell lysates while APP α , APP β , and A β were detected in the media of NT2N neurons. Surprisingly, in the presence of BFA, not only APP_{FL} but also APP β and A β continued to be recovered from NT2N cell lysates. The effectiveness of BFA was verified by the fact that the secretion of APP α , APP β , and A β into the medium was completely abolished in its presence.

Cell lysates and medium from the cells were then analyzed using an ELISA system which quantitatively distinguishes between A β ₄₀ and A β ₄₂ to ascertain whether alteration of A β levels by BFA was the result of altered levels of A β ₄₀, A β ₄₂ or both. Suzuki et al. 1994 *Science* 264:1336-1340. Results from the ELISA correlated with the immunoprecipitation data in that BFA abolished secretion of A β into the medium and reduced overall expression of intracellular A β by approximately 60%. However, it was the complete loss of A β ₄₀ that accounted for this decrease. A β ₄₂ was largely unaffected by BFA treatment. Thus, γ -secretases appear to be active in the endoplasmic reticulum, but only to yield A β ₄₂. Further, it is believed that A β ₄₂ is preferentially generated in the ER while A β ₄₀ is generated in more distal components of the exocytic pathway.

Since BFA treatment results in retention of all newly synthesized proteins in the ER, additional experiments were conducted to confirm that the generation of A β ₄₂ in the ER was not due to retention of newly synthesized proteases not being delivered to their proper site of action. Accordingly, an ER-retention signal was placed in APP695 in which the third and fourth amino acids from the C-terminus were changed to lysine (APP695_{ΔKK}). This lysine motif is sufficient to retain heterologous transmembrane proteins in the ER and intermediate

compartment. In these experiments, recombinant Semliki Forest virus (SFV) vectors to express APP695_{AKK}. Previous studies have shown that despite high levels of SFV-mediated APP expression, SFV infected NT2N cells display a high degree of fidelity in processing APP (Wertkin et al. 1993 *Proc. Natl. Acad. Sci. USA* 90:9513-9517; Turner et al. 1996 *J. Biol. Chem.* 271:8966-8970). Furthermore, it was found that cytopathic effects of SFV infection in NT2N cells as measured by LDH release do not develop until more than 48 hours after infection. To determine if APP695_{AKK} was in fact retained in the ER, indirect immunofluorescence microscopy was used to monitor both intracellular and cell surface distribution of SFV-expressed APP695 and APP695_{AKK}. While intracellular levels of APP695 and APP695_{AKK} appeared similar, only APP695 was expressed on the cell surface. BFA treatment blocked surface expression of APP695. In addition, APP695_{AKK} did not acquire resistance to endoglycosidase H digestion, a further indication that APP695_{AKK} is retained in the ER.

By retaining APP695_{AKK} in the ER, it was then determined whether A β is generated in this compartment without concomitant BFA-induced accumulation of other cellular proteins. SFV-infected NT2N cells were metabolically labeled overnight, and A β immunoprecipitated from the medium and cell lysate. It was found that ER retention of APP by the KK retention signal efficiently blocked A β secretion, yet failed to block all intracellular A β biosynthesis. Western blot analysis of intracellular and secreted APP showed that APP695 and APP695_{AKK} were expressed to comparable levels. As with BFA treatment, cells expressing APP695_{AKK} produced 40% of the total intracellular A β generated from APP695. Quantitative ELISA showed that this reduction was accounted for by the loss of A β ₄₀. A β ₄₂ levels were not affected. Thus, A β ₄₂ is cleaved from APP while still in the ER.

APP β recovered from BFA treated cells was found to migrate with an accelerated electrophoretic mobility compared to APP β from non-treated cells, suggesting that this fragment may have been derived from immature APP. To confirm that

incomplete maturation of APP was the cause of the shift in electrophoretic mobility of the APP β fragment generated in the presence of BFA, NT2N cells were metabolically labeled with [³⁵S]methionine in the presence or absence of BFA. APP β immunoprecipitated from the cell lysate was then incubated with N-glycosidase F (Nglyc F), an enzyme that removes N-linked carbohydrate chains. APP β from BFA-treated NT2N neurons migrated more quickly than APP β recovered from untreated cells. After digestion with Nglyc F, APP β demonstrated a mobility downshift in SDS-PAGE. However, APP β from BFA treated cells still migrated faster than APP β from non-treated cells despite enzymatic removal of all N-linked carbohydrate chains. Thus, the increased electrophoretic mobility of APP β in the presence of BFA cannot be accounted for solely by differences in N-linked carbohydrate processing.

In addition to N-linked glycosylation, however, APP undergoes a variety of post-translational modifications, including the addition of O-linked carbohydrate chains. Therefore, both N- and O-linked carbohydrate chains were removed from immuno-precipitated APP β by simultaneous digestion with Nglyc F, O-glycosidase, and neuraminidase. Fully deglycosylated APP β co-migrated with APP β recovered from BFA treated NT2N neurons. Furthermore, combined BFA inhibition and deglycosylation did not induce a greater mobility shift than either of these treatments alone. Accordingly, APP β generated from BFA treated NT2N neurons is believed to represent β -secretase processing of immature (non-glycosylated) APP_{FL} in a pre-Golgi compartment.

To further verify that β -secretase cleavage occurs early in the biosynthetic pathway of NT2N neurons, an alternative non-pharmacological method to block protein transport from the ER to the Golgi was employed. Incubation of cultured cells at 15°C has been shown to inhibit newly synthesized proteins from exiting the intermediate compartment (Saraste, J. and Kuismanen, E. 1984 *Cell* 38:535-549; Saraste et al. 1986 *Proc. Natl. Acad. Sci. USA* 83:6425-6429; Schweizer et al. 1990 *Eur. J. Cell. Biol.* 53:185-196). In these

experiments, NT2N cells were incubated at 15°C for 16 hours. Only the immature form of APP_{FL} was present after a 16 hour incubation at 15°C as indicated by its sensitivity to Endoglycosidase H (Endo H) digestion, suggesting that it is not transported to the Golgi apparatus under these conditions. By contrast, incubation of the NT2N cells at 37°C yielded both immature and fully processed APP_{FL}. As expected, the immature APP_{FL} was Endo H sensitive, while the mature forms of APP_{FL}, having acquired post-translational modifications after exiting the ER, were Endo H resistant. In addition, secreted forms of APP were not detected in cells maintained at 15°C, further substantiating the effectiveness of the temperature block. Significantly, continuous production of intracellular APP β was observed at 15°C despite the fact that the secretion of APP ectodomain is completely abolished. Thus, these data also support the endoplasmic reticulum (ER)/intermediate compartment (IC) of NT2N neurons as a β -cleavage site.

The processing of wild-type APP695 and APP695 bearing the ER retrieval motif (APP695_{AKK}; Jackson et al. 1990 *EMBO J.* 9:3153-3162; Jackson et al. 1993 *J. Cell Biol.* 121:317-333) in the NT2N cells was also examined with regard to cleavage of APP β . To determine whether or not APP β can be produced from APP695_{AKK}, wild-type APP695 and APP695_{AKK} were separately expressed in NT2N neurons by infection with SFV vectors bearing these constructs. Following infection, duplicate wells containing wild-type APP695 infected cells were also treated with 20 μ g/ml BFA. The [³⁵S]methionine labeled cell lysates and the media were then sequentially immunoprecipitated with the antibodies 53 and Karen. Only the immature form of APP_{FL} was detected from cells expressing APP695_{AKK}. Significantly, intracellular production and secretion of APP β was not affected by genetic targeting of APP to the ER. Furthermore, it was found that unlike inhibition with BFA that eliminates transport of all proteins from the ER to the Golgi, specific retrieval of full-length APP695_{AKK} to the ER allowed the APP β fragment generated in the ER/IC to be transported to the Golgi complex for modification before secretion. Thus, it is believed that

once the ER retention motif is cleaved from the APP β fragment, it can then be transported to the Golgi complex for further maturation and subsequent secretion.

Generation of A β in the ER of NT2N neurons identifies these cells as a unique system in which to study amyloidogenic processing of APP and its role in the pathogenesis of AD. Mutations in both the APP gene and the recently identified presenilin genes are believed to cause AD by altering APP processing in ways that lead to the production of more amyloidogenic forms of A β (i.e., A β_{42} ; Scheuner et al. 1996 *Nature Med.* 2:864-870). Recently, in both non-neuronal and neuronal cells (including the NT2N neurons used in this study), the presenilin proteins have been localized to the ER (Cook et al. 1996 *Proc. Natl. Acad. Sci. USA* 93:9223-9228; Kovacs et al. 1996 *Nature Med.* 2:224-229; Thinakaran et al. 1996a. *Neuron* 17:181-190). Thus, the identification of amyloidogenic processing that occurs within the ER of neurons suggests that direct or indirect interaction may occur between the presenilins and APP. Furthermore, the mutations in the presenilin genes may alter this interaction in a manner that leads to increased production of APP β and ultimately A β_{42} . Accordingly, the identification of these secretase pathways in the ER of neuronal cells will permit the examination of the effects of both FAD-linked mutations occurring in the APP as well as the presenilin genes on the processing of APP in the ER. Further agents which modulate APP processing by increasing or decreasing production of APP β and A β_{42} can be identified by determining their effects on levels of APP β and A β_{42} produced by β - and γ -secretases in the ER of neuronal cells such as NT2N cells. Levels of peptides produced by this pathway can be routinely determined in accordance with teachings provided herein. Agents identified by this method to inhibit levels of APP β and/or A β_{42} produced by these enzymatic pathways may be useful in treating Alzheimer's disease while agents which increase levels of APP β produced by this pathway may be causative factors in the development of Alzheimer's disease.

The following nonlimiting examples are provided to

further illustrate the present invention.

EXAMPLES

Example 1 Cell Culture

NT2 cells derived from a human embryonal carcinoma
5 cell line (Ntera 2/cl.D1) were grown and passage twice weekly
in Opti-Mem (Life Technologies, Inc.) supplemented with 5%
fetal bovine serum (FBS) and penicillin/streptomycin (P/S) in
accordance with procedures described by Pleasure et al. 1992 *J.*
Neurosci. 12:1802-1815 and Pleasure, S. J. and Lee, V. M.-Y.
10 1993 *J. Neurosci. Res.* 35:585-602. To begin differentiation,
 2.5×10^6 cells were seeded in a 75 cm² (T75) flask and fed with
Dulbecco's modified Eagle's medium (DMEM) HG (Life
Technologies, Inc., Gaithersburg, MD) containing 10 μ M retinoic
acid, 10% FBS and P/S twice weekly for 5 weeks. The cells in
15 a single T75 flask were then replated at a lower density in 2
 $\times 225$ cm² (T225) flasks for 10 days (Replate 1 cells). NT2N
neurons with greater than 99% purity were then obtained by
enzymatic treatment and mechanical dislodgment of Replate 1
cells and replated at a density of 6×10^6 cells per 10 cm dish
20 previously coated with polylysine and Matrigel as described by
Pleasure et al. 1992 *J. Neurosci.* 12:1802-1815. The NT2N
neurons were maintained in medium consisting of one part
conditioned medium and one part DMEM HG containing 10% FBS and
P/S. For experiments involving the incubation of NT2N neurons
25 at 15°C for 16 hours, regular medium containing DMEM HG and 10%
FBS was replaced by DMEM HG containing 25 mM HEPES, 10% FBS,
and P/S. Cultures of NT2N neurons were used for experiments
when they were between 3 to 4 weeks old. CHO695 cells were
grown and passaged three times per week in α -MEM (Life
30 Technologies, Inc., Gaithersburg, MD) supplemented with 10% FBS
and P/S. M17 cells were grown and passage once per week in
Opti-mem (Life Technologies, Inc., Gaithersburg, MD) containing
10% iron enriched calf serum and P/S.

**Example 2 Metabolic Labeling, Gel Electrophoresis,
Immunoblotting and Quantitation**

Cultured NT2N neurons were starved in methionine-free DMEM HG (Life Technologies, Inc., Gaithersburg, MD) for 30 minutes prior to incubation in fresh methionine-free DMEM HG containing 0.5 mCi/ml of [³⁵S]methionine (sp act. 1000 Ci/mmol; NEN-DuPont, Boston, MA). For steady-state labeling studies, NT2N neurons were labeled with [³⁵S]methionine continuously for 16 hours. For pulse-chase studies, cells were labeled with [³⁵S]methionine for 1 hour, washed twice with methionine-containing DMEM, and then chased in the same medium for 0 to 24 hours. APP_{FL}, APP α and APP β were separated on 7.5% Laemmli SDS-PAGE gels, and A β and p3 were separated on 10/16.5% step-gradient Tris-Tricine gels. These gels were either stained with Coomassie Brilliant Blue R (Pierce, Rockford, IL) and dried or transferred to nitrocellulose membranes and dried prior to exposure on PhosphorImager plates (Molecular Dynamics Inc., Sunnyvale, CA) for 3-5 days. The nitrocellulose replicas containing the immunoprecipitates were further probed with different antibodies in accordance with procedures described by Wertkin et al. 1993 *Proc. Natl. Acad. Sci. USA* 90:9513-9517. Quantitation of bands in the autoradiogram was performed using the ImageQuant software (Molecular Dynamics Inc. Sunnyvale, CA) in accordance with procedures described by Turner et al. 1996 *J. Biol. Chem.* 271:8966-8970. Radiolabeled proteins in SDS-PAGE gels and nitrocellulose replicas were also analyzed by standard autoradiographic methods. All experiments were repeated between 3 and 6 times.

**Example 3 Sample Preparation and Serial
Immunoprecipitations**

Cell lysates were prepared in accordance with procedures described by Golde et al. 1992 *Science* 255:728-730. Protein concentration was determined by the bicinchoninic acid procedure (Pierce, Rockford, IL). Media were centrifuged at 100,000 x g for one hour at 4°C before immunoprecipitation. Both cell lysates and media were precleared with protein A-

Sepharose (Pharmacia Biotech, Piscataway, NJ) in RIPA for one hour at 4°C. After recentrifugation at 15,000 x g for one minute, the supernatants were rocked overnight at 4°C with fresh protein A-Sepharose and the appropriate primary antibody.

5 After collecting the immunoprecipitates by recentrifugation at 15,000 x g for 1 minute, the supernatants were used in a second round of immunoprecipitation with fresh protein A-Sepharose and a different primary antibody.

Example 4 Trypsin Treatment of NT2N Neurons

10 NT2N neurons were metabolically labeled with 0.5 mCi/ml [³⁵S]methionine for 16 hours in accordance with the procedure set forth in Example 2. After rinsing the cultures twice with PBS, the NT2N neurons were incubated on ice for 20 minutes with PBS, with 10 µg/ml of trypsin in PBS alone (Life Technologies, Inc., Gaithersburg, MD), or with 10 µg/ml trypsin

15 and 0.1% Triton X-100 in PBS. Following this treatment, trypsin was inactivated by the addition of 100 µg/ml soybean trypsin inhibitor. The cells were then washed with PBS, scraped into cell lysis buffer, and processed for

20 immunoprecipitation as described in Example 3.

Example 5 Brefeldin A Treatment of NT2N Neurons and Deglycosylation of Immunoprecipitated APP β

NT2N neurons were pretreated with 20 µg/ml of BFA for 1 hour before the addition of 0.5 mCi/ml of [³⁵S]methionine to

25 the cultures for 16 hours in the absence or presence of BFA. The cell lysates and media were processed for immunoprecipitation as described in Example 3. For deglycosylation of APP β , the immunoprecipitates containing APP β were washed twice in sodium phosphate buffer (20 mmol/l, pH

30 7.2) and boiled for 2 minutes in 10 µl of 1% SDS. The samples were then boiled for an additional 2 minutes after adding 90 µl of the sodium phosphate buffer with sodium azide (10 mmol/l), EDTA (50 mmol/l), and n-Octylglucoside (0.5% w/v). After this denaturation step, deglycosylation was initiated by the

35 addition of 2 mU Neuraminidase (Arthrobacter; Boehringer

Mannheim, Indianapolis, IN), 2.5 mU O-Glycosidase (Boehringer Mannheim, Indianapolis, IN), and 0.4 U N-Glycosidase F (Boehringer Mannheim, Indianapolis, IN). The samples were then incubated at 37°C for 18 hours and deglycosylated APP β was run
5 on 7.5% SDS-PAGE gels as described in Example 2.

For Endoglycosidase H (Endo H) sensitivity test, cell lysates and media were immunoprecipitated with Karen in accordance with procedures described in Example 3. Immunoprecipitates were recovered in 100 μ l 60 mM phosphate
10 buffer, pH 5.7 with 1% SDS. The samples were then split in half (50 μ l each), and incubated with 4 μ l Endo H (Boehringer Mannheim, Indianapolis, IN) or vehicle at 37°C for 18 hours. The samples were then run on 7.5% SDS-PAGE gels in accordance with procedures described in Example 2.

15 **Example 6 Antibodies for Immunoprecipitation and Immunoblotting**

Karen is a goat polyclonal antisera raised to the large secreted N-terminal fragment of APP, and antibody 53 is a rabbit polyclonal antisera raised to a synthetic peptide
20 corresponding to the amino acid sequence SEVKM. Antibody 53 binds specifically to the free C-terminus of APP β as disclosed by Howland et al. 1995 *Neurobiol. Aging* 16:685-699. Antibody 369W is a rabbit polyclonal antiserum raised to a synthetic peptide corresponding to the last 45 amino acid residues at the
25 C-terminus of APP. Also used in this study were three monoclonal antibodies (MAb) to A β that are specific for residues 1-17 (6E10; Kim et al. 1988 *Neurosci. Res. Commun.* 2:121-130); residues 1-10 (Ban50; Suzuki et al. 1994 *Science* 264:1336-1340) and residues 18-25 (4G8; Kim et al. 1988
30 *Neurosci. Res. Commun.* 2:121-130).

- - **Example 7 Preparation of SFV bearing pSFV-I(APP695) and pSFV-1(APP695_{AKK})**

The di-lysine motif was introduced into APP695 by standard PCR site-directed mutagenesis of pSFV-1(APP695) using
35 primers 5'-CGAAAACCAACCGTGGAGCTCCTT-3' (SEQ ID NO: 1) and 5'-

TTAACCCGGGCTAGTTCTGCTTCTTCTCAAAGAACTTGT-3' (SEQ ID NO: 2). The mutation containing PCR fragment was isolated by digestion with Bsm-1 and Xma-1, then ligated into pSFV(APP695) to yield pSFV(APP695_{AKK}). All pSFV-1 constructs, including a pSFV helper
5 plasmid with SFV structural genes, were linearized by digestion with Spe-1 and then used as a template for RNA synthesis with SP6 RNA polymerase. Co-electroporation of RNA from the expression and helper plasmids into BHK cells yielded infectious, replication-defective virus that was harvested 24
10 hours later in accordance with procedures described by Liljestrom, P. and Garoff, H. 1991 *Bio/Technology* 9:1356-1361. Accurate determination of viral stock titers were made in accordance with procedures described by Cook et al. 1996 *Proc. Natl. Acad. Sci. USA* 93:9223-9228. For all infection
15 experiments approximately 1×10^6 NT2N neurons per 35 mm dish were infected in serum free medium at a multiplicity of infection (MOI) of 7-10. When called for, 20 μ g/ml BFA was added after the completion of the infection step.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Virginia Lee, Robert Doms

(ii) TITLE OF INVENTION:

Methods of Identifying Modulators of Amyloid Precursor
Protein Processing in the Endoplasmic
Reticulum/Intermediate Compartment of NT2N Cells

(iii) NUMBER OF SEQUENCES: 2

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CGAAAACCAC CGTGGAGCTC CTT

23

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2: - - - - -

TTAACCCGGG CTAGTTCTGC TTCTTCTCAA AGAACTTGT 39

What is Claimed is:

1. A method of identifying agents which increase or decrease processing of amyloid precursor protein into amyloid β peptides found in neuritic plaques and vascular deposits that accumulate in brains of patients with Alzheimer's disease comprising:

contacting NT2N cells with a compound or agent suspected of increasing or decreasing amyloid precursor protein processing; and

10 measuring levels of amyloid β peptides formed in the endoplasmic reticulum of the cells.

2. A method of diagnosing Alzheimer's disease in a patient comprising detecting in the patient an agent identified to 15 increase processing of amyloid precursor protein into amyloid β peptides found in neuritic plaques and vascular deposits that accumulate in brains of patients with Alzheimer's disease in accordance with the method of claim 1.

3. A method of inhibiting processing of amyloid precursor 20 protein into amyloid β peptides found in neuritic plaques and vascular deposits that accumulate in brains of patients with Alzheimer's disease comprising administering to the patient an agent which decreases processing of amyloid precursor protein into amyloid β peptides found in neuritic plaques and 25 vascular deposits that accumulate in brains of patients with Alzheimer's disease wherein said agent is identified in accordance with the method of claim 1.

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/20212

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 33/567; A01N 37/18; A61K 38/00

US CL : 435/7.21, 514/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.21, 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,385,915 (BUXBAUM ET AL) 31 January 1995, see entire document.	3
X	US 5,538,983 (BUXBAUM ET AL) 23 July 1996, see entire document.	3
Y	US 5,262,332 (SELKOE) 16 November 1993, see entire document.	1, 2
Y	US 5,547,841 (MAROTTA ET AL) 20 August 1996, see column 3, lines 7-14 and claims 1-3.	1, 2

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

29 JANUARY 1998

Date of mailing of the international search report

04 MAR 1998

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/20212

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HAASS et al. The Swedish mutation causes early-onset Alzheimer's disease by beta-secretase cleavage within the secretory pathway. Nature Medicine, December 1995, Vol. 1, No. 12, pages 1291-1296, see entire document.	1, 2
Y	WERTKIN et al. Human neurons derived from a teratocarcinoma cell line express solely the 695-amino acid amyloid precursor protein and produce intracellular beta-amyloid or A4 peptides. Proc. Natl. Acad. Sci. USA, October 1993, Vol. 90, pages 9513-9517, see entire document.	1, 2
Y	TURNER et al. Amyloids beta40 and beta42 are generated intracellularly in cultured human neurons and their secretion increases with maturation. The Journal of Biological Chemistry, 12 April 1996, Vol. 271, No. 15, pages 8966-8970, see entire document.	1, 2
Y	MARTIN et al. Intracellular accumulation of beta-amyloid in cells expressing swedish mutant amyloid precursor protein. The Journal of Biological Chemistry, 10 November 1995, Vol. 270, No. 45, pages 26727-26730, see entire document.	1, 2

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/20212

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG, EMBASE, DERWENT WPI, JPIO, MEDLINE, BIOSYS, CAB ABSTRACTS.

search terms: amyloid, beta-amyloid, intracellular, NT2T cells, neuron, neuronal, modulate, increase, decrease, inhibit, assay, screen.